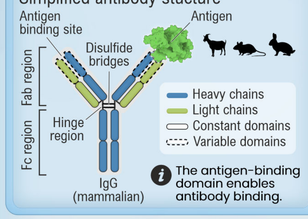
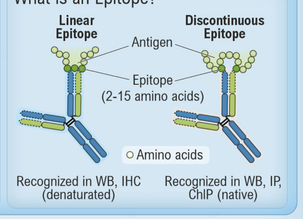
Western Blotting: Give Your Blots a Chance to Improve

Key Things to Know Before Starting Western Blotting

Basic Knowledge





Target Protein Sequence. How do I choose the right antibody for my protein?

The antibody-eliciting sequence must be present in the target protein!

Example: >sp|P83755|PSBA_ARATH Photosystem II protein D1

Signal peptide (cleaved off):

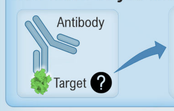
MTAILERRESESLWGRFCNWITSTENRLYIGWFGVLMIPTLLTATSVFIIAFIAAPP
VDIDGIREPVSGSLLYGNNIISGAIIPTSAAIGLHFYPIWEAASVDEWLYNGGPYEL
IVLHFLLGVACYMGREWELSFRLGMRPWIAVAYSAPVAAATAVFLIYPIGQGSFSD
GMPLGISGTFNFMIVFQAEHNILMHPFHMLGVAGVFGGSLFSAMHGSLVTSSLIRE
TTENESANEGYRFGQEEETYNIVAAHGYFGRLIFQYASFNNSRSLHFFLAAWPVV
GIWFTALGISTMAFNLNGFNFNQSVVDSQGRVINTWADIINRANLGMEVMHERNA

PsbA

Ab to C-terminal

Antibody binding to a linear epitope requires >70% linear sequence identity

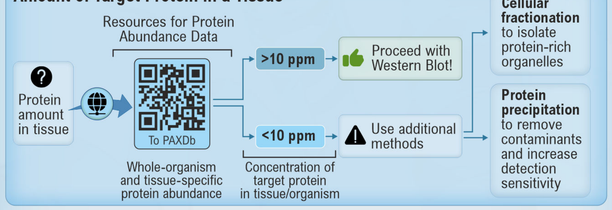
How much do you know about the protein you aim to detect?



Protein check recommended by Agrisera

- Biochemical properties: isoelectric point pI, solubility etc.
- Expression level
- Species, organ, and tissue localization
- Cellular localization

Amount of Target Protein in a Tissue

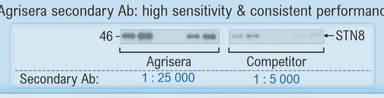


Protein Transfer: PVDF *vs.* Nitrocellulose Membranes

Feature	PVDF	Nitrocellulose
Protein binding	High; hydrophobic	Moderate; hydrophobic & electrostatic
Durability	Strong; air-dried/re-wetted	Brittle & fragile; air-dried/re-wetted
Sensitivity	Ideal for low-abundance proteins	Suitable for high-abundance proteins
Pre-treatment	Methanol pre-wetting needed	No pre-wetting needed
Protein size	Broad range	Not for low MW proteins (< 20 kDa)

Secondary Antibodies

- Use at high dilutions to reduce background, cross-reactivity
- Max incubation time = 1 h/RT **– do not exceed!**
- Agrisera secondary Ab: high sensitivity & consistent performance



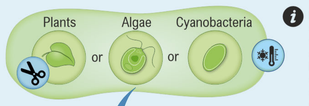
To Agrisera's Guide and products

4 Key Factors for Choosing Antibodies (Recommended by Agrisera)

- Species Reactivity: Confirmed experimentally or predicted based on sequence conservation
- Validation Data: Reliable antibodies must be validated in endogenous samples for accuracy
- Tested Applications: Each technique requires a separate validation
- Publication Record: Citations on supplier websites add credibility and guide your selection

Western Blotting Workflow

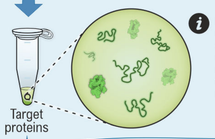
1 Sample Collection
Careful and prompt collection of tissues or cells to minimize protein degradation.



Even short delays during sample collection can lead to protein degradation and altered results

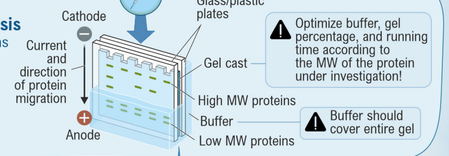
2 Protein Extraction
Extraction of proteins using protocols from the Agrisera website (also see, Agrisera Poster 8).

To protein extraction protocols



Extraction under denaturing conditions usually causes most proteins to lose their native structure

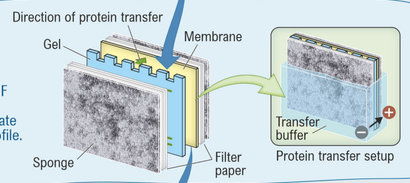
3 Gel Electrophoresis
Separation of proteins by molecular weight using SDS-PAGE, with optimized gel percentage and running time.



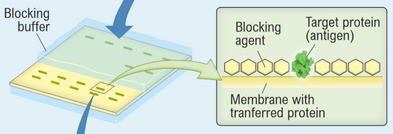
Optimize buffer, gel percentage, and running time according to the MW of the protein under investigation!

Buffer should cover entire gel

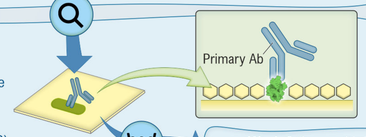
4 Protein Transfer
Transfer of proteins from the gel to PVDF or nitrocellulose membrane to replicate the gel's protein profile.



5 Blocking
Incubation of the membrane in a blocking buffer (protein or protein-free) to prevent non-specific Ab binding (ON at 4°C or 1 h at RT).

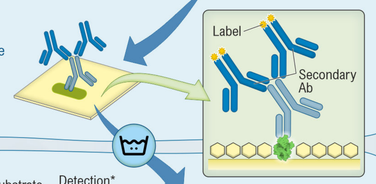


6 Primary Antibody Incubation
Incubation of the membrane with a primary Ab specific to the target protein (ON at 4°C, 1 h at RT, or 0.5-1 h at 37°C).



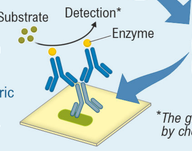
7 Wash 3 times with TBST or PBST buffer to remove unbound primary Ab.

8 Secondary Antibody Incubation
Incubation of the membrane with a secondary Ab conjugated to an enzyme (ALP, HRP) or fluorescent dye for up to 1 h at RT.



9 Wash 3 times with TBST or PBST buffer to remove unbound secondary Ab.

10 Detection
Visualization of the protein bands using chemiluminescence, fluorescence, or colorimetric methods, depending on the required sensitivity.



**The graphic shows detection by chemiluminescence only*

Western Blot Tips & Troubleshooting

Tips

General: Get a correct band first **before** testing variations!

For Beginners

- Samples: WT & specific mutant
- Protein load: >50 µg/well
- Blocking: 5% nonfat milk/BSA, 1 h/RT
- Primary Ab: 1:500–1:1000, ON/4°C
- Secondary Ab: 1:25000, 1 h/RT
- Always record results!

For Advanced Users

- Review protocol and perform optimization!
- One protocol doesn't fit all antibodies

Complex issues? Contact Agrisera support@agrisera.com

No Signal is Detected in a Total Cell Extract?

- No signal detected
- Use specific organ or developmental stage! → Plant / Leaves
- Fractionate extract to isolate certain organelles → Chloroplast

Fractionation concentrates target proteins, enhancing detection of non-cytoplasmic proteins, particularly those with low expression.

Check cellular expression data/ePlant

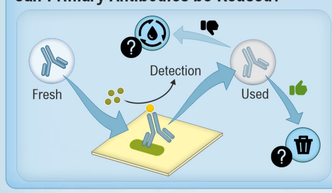
Transfer Conditions Based on Protein Size

Protein Size	Challenges	Recommended conditions: Membrane	MeOH	SDS	Transfer
High MW Proteins (>20 kDa)	Retained in gel; longer transfer time	0.45 µm	Low (10%)	Increased	Wet (tank)
Low MW Proteins (<20 kDa)	Easily migrates out of gel; shorter transfer time	0.20 µm	High (20%)	Decreased	Semi-dry

MeOH is toxic! EtOH is a safer, equally effective alternative to MeOH

Perform wet and semi-dry transfers under cold conditions to prevent heat buildup

Can Primary Antibodies be Reused?



Answer: It is antibody-dependent, but...

Re-use is generally not recommended, especially in quantitative experiments

Discard in most cases to avoid signal loss

How to Determine Gel Loading?

Loading Method	Recommendation	Notes
µg of Protein (*)	Recommended	Best for accurate loading
µg of Chlorophyll (**)	Recommended	Suitable for plant samples
Volume (µl)	Not recommended	Can lead to inconsistent results

(*) For example, there are reagents which allow fast protein quantification and are compatible with detergents, salts, and chelators present in the extraction buffer

(**) For photosynthetic tissues

Quick Protein Transfer Quality Control

Good transfer

- No bands on Coomassie gel (protein moved to the membrane)
- Even, reversible protein staining (e.g., Ponceau S)
- Clear & sharp bands after antibody detection

Poor transfer

- Faint or visible bands in gel → Incomplete transfer
- Uneven band disappearance → Transfer issue
- Smearing or distorted bands

Troubleshoot transfer condition!

To further details on protein transfer

Is Sample Denaturation Above 90°C a Golden Standard?

Answer: Not always! High temperatures can cause protein aggregation, and signal loss in Western Blot, while membrane proteins may never enter the gel

For total cell extracts from plant tissues & algal cells Agrisera recommends: 70°C 5–10 min

Key Considerations

- **Check antibody datasheets:** Some antibodies prefer native proteins, while others require target protein denaturation
- **Know your antibody's target:** Does it bind to a native or denaturated conformation?

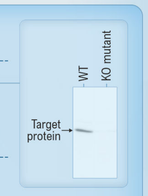
Antibody Validation Checklist

Genetic Validation (Direct approach)

- KO/KD Control: Target protein signal disappears or decreases in KO/KD samples
- IP-MS: Confirms antibody binds the correct protein

Biochemical & Experimental Validation (Indirect approach)

- Correct MW: Band appears at expected MW, considering modifications
- Low Cross-Reactivity: Minimal non-specific binding
- Cellular Localization: Signal detected in the correct cellular fraction
- Expression-Based Validation: Signal changes with known regulatory conditions
- Independent Antibody Confirmation: Another validated antibody detects the same band
- Pre-immune Serum Control: No/minimal signal in pre-immune serum
- Peptide Neutralization: Signal reduces with target peptide pre-incubation



How to Cite an Antibody? Why it Matters?

Provide the following: **Antibody name** + **Product number** + **Supplier**
Example: Anti-PsbA AS05 084 Agrisera

Other Key Western Blot Details to Report:

- Protein load per well
- Primary antibody dilution
- Blocking agent, concentration, and incubation time
- Membrane type

It helps increase reproducibility!

How to Get a Strong Signal from a Weak Antibody?

Answer: Optimization

Key Factors for Signal Enhancement

- Sample type & fractionation: Ensure proper sample preparation
- Transfer conditions: Confirm efficient protein transfer
- Antigen-purified antibodies: Improve specificity and sensitivity
- Protein load per well: Optimize for best signal without oversaturation
- Detection reagents: Choose an appropriate one for a given target protein abundance

To Agrisera's Western Blot Resources

Western Blotting: Give Your Blots a Chance to Improve. For more detailed information, scan QR codes and check specific references and methods therein. Send questions, comments, and suggestions to Joanna Porankiewicz-Asplund (joanna@agrisera.com). **Abbreviations:** AA, amino acids; Ab, antibody; BCA, bicinchoninic acid; BSA, bovine serum albumin; ChIP, chromatin immunoprecipitation; EtOH, ethanol; HRP, horseradish peroxidase; IgG, immunoglobulin G; IHC, immunohistochemistry; IP, immunoprecipitation; KO/KD, knockout/knockdown; MeOH, methanol; MW, molecular weight; ON, overnight; PAXDb, protein abundance database; PBST, phosphate-buffered saline with Tween® 20 detergent; PSI, photosystem I; ppm, parts per million; PVDF, polyvinylidene fluoride; RT, room temperature; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBST, tris-buffered saline with Tween® 20 detergent; WB, Western Blot; WT, wild type. **Notes:** Protein complexes were generated using *Protein Imager* using coordinates deposited at PDB with IDs 3jcu and 7oui. **Acknowledgements:** We thank Anna Węgrzyn, David Alabadi, Dhruv Agrawal; Edith Kalén, Louice Lindroth, Mariana Barber, Mika Teranishi, Pushan Bag, Sebastian Hoernstein, and Ximena Anleu Gil for valuable comments and corrections. We are highly grateful to Agrisera for sponsoring poster design, printing, and free distribution at conferences around the world. Citation: Porankiewicz-Asplund J, Shevela D, et al (2025) Western Blotting: Give Your Blots a Chance to Improve, *Agrisera Educational Poster 9*: doi:10.6084/m9.figshare.29086685.

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Agrisera Antibodies

www.agrisera.com

Poster 9 - Western Blotting: Give Your Blots a Chance to Improve, 2025

© 2025 Joanna Porankiewicz-Asplund[1], Dmitry Shevela[2] & Hundreds of researchers who shared Western Blot data over 25 years[3]

[1]Agrisera AB, Sweden; [2]Umeå University, Sweden; [3]Universities worldwide

Plant Meetings Calendar
Sign up your meeting, or check what is coming!
https://plantae.org/events-calendar/